

THE EFFECTS OF IONS ON MUCIN RHEOLOGY AND ORGANIZATION

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ABSTRACT

Kala Ndi Nwachukwu: The effects of ions on mucin rheology and composition
(Under the direction of Robert Tarran and David Hill)

Increased mucus thickness is a common symptom of pulmonary diseases, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) and also occurs in chronic cigarette smokers. The thickening of the mucus layer is caused by a defect in the cystic fibrosis transmembrane conductance regulator (CFTR), which causes an imbalance of ion transport, i.e. reduced CFTR mediated anion secretion, dehydration of the mucus layer, and poor mucociliary clearance. Inhaled pathogens become trapped within the mucus layer, resulting in increased infection and inflammation. Here, we present data demonstrating the effects of ions that play a role in mucus layer homeostasis and their effects on mucin composition and rheology. We demonstrate that extracellular CaCl_2 increases complex mucin viscosity, while MgCl_2 has no effect. We also provide evidence indicating that CaCl_2 effects the ability of mucins to interact/bind to glycoproteins. Furthermore, we show that HCO_3^- and pH effect mucin rheology, whereas lower pH and higher HCO_3^- levels result in a higher complex viscosity. While the influence of CaCl_2 is not currently known, our data suggest that in excess it may contribute to the viscoelastic properties of mucus.

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LIST OF ABBREVIATIONS AND SYMBOLS

ASL	Airway surface liquid
BAPTA	1,2-bis(o-aminophenoxy)ethane- <i>N,N,N',N'</i> -tetra-acetic acid
BSM	Bovine submaxillary mucin
CaCl ₂	Calcium chloride
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
COPD	Chronic obstructive pulmonary disorder
EGTA	Ethylene glycol tetra-acetic acid
ENaC	Epithelial sodium channel
Eta (η^*)	Complex viscosity
FRAP	Fluorescence recovery after photobleaching
HCO ₃ ⁻	Bicarbonate
MgCl ₂	Magnesium chloride
MSD	Mean squared displacement
MUC5B	MUCIN 5B

MUC5AC	MUCIN 5AC
PCL	Periciliary liquid
PGM	Porcine gastric mucin
SPLUNC1	Short palate lung and nasal epithelial clone 1
WGA	Wheat germ agglutinin
WT%	Weight percent

CHAPTER 1: AIRWAY MUCUS AND MUCIN DEVELOPMENT IN CYSTIC FIBROSIS

Introduction

Airway luminal mucus is a solution that consists of lipids, glycoproteins, secreted polypeptides, DNA, proteins, cells, ions, water, and cellular debris. Mucus is a secreted viscoelastic fluid that lines the epithelium of the eye, airways, gastrointestinal and urogenital tracts (Georgiades et al. 2013). The primary role of airway mucus is to serve as the first line of defense against inhaled foreign molecules including pathogens/toxins that enter the airway. This mechanism is known as mucociliary clearance (Rogers et al. 2007). The airway surface liquid (ASL) consists of two distinct layers. The first layer is the mucus layer, which contains large gel-forming mucins that trap inhaled particles for removal from the lung by mucociliary clearance (Widdicombe et al. 1995). Below this mucus layer is the periciliary liquid layer (PCL), which is a more dilute liquid layer that surrounds the cilia and provides hydration to the epithelium and enables mucus transport (Button et al. 2012). Membrane-bound mucins within the PCL are tethered to the cilia and apical surface of airway epithelial cells. They form a well connected mesh gel-network that prevents inhaled toxins from entering the PCL.

In healthy individuals, mucus percent (wt%) solids are 1.5-2.5%, whereas it increases to 4-5% in chronic obstructive pulmonary disease (COPD) patients and 6-8% in cystic fibrosis (CF) patients” (Hill et al. 2014). The remaining ~95% of mucus is water (Cavaliere et al. 1989). Mucus and sputum are terms that are often used loosely and

interchangeably, however, sputum and mucus differ in clearance from the lungs. Sputum or “spit” is a combination of mucus and saliva, which is normally cleared by coughing, whereas, mucus is cleared by mucociliary clearance (Rubin et al 2014).

A study by Williams et al. suggested that airway inflammation, mucus hypersecretion, and impaired mucociliary clearance are characteristics shared by nearly all obstructive pulmonary diseases (Williams et al. 2006). Excessive build-up of mucus in the ASL and disrupted ion transport perturb PCL volume, which disrupts ciliary beat frequency thus contributing to poor mucociliary clearance (Derichs et al. 2011). Disrupted ion transport is due to a defective CFTR anion channel activity and/or epithelial sodium channel (ENaC) hyperactivity (Derichs et al. 2011). Mucociliary clearance can be affected by numerous factors including: the volume of mucus, ionic composition of the mucus and the pH of the fluid secreted in the airway. A certain viscoelasticity combination is required in mucus for effective cilia beating (Rogers et al. 2007).

The production of mucus is very intricate and involves synthesis of the mucus, packaging and exocytosis. Mucins then begin to swell and are transported and discharged into the lumen (Yang et al. 2013).

Section 1.1 Mucin and mucus gel-forming properties

The major gel-forming components of mucus are glycoproteins known as mucins. Mucins are negatively charged, glycosylated proteins that are continuously synthesized and secreted to replenish the mucus layer. Mucins are monomeric structures that tether together to make up a mucin network. Mucins contain high amounts of saccharides, in-particular O-glycans. Mucins also have cysteine-rich domains where no glycosylation is

present. They also have hydrophobic properties (Shankar et al. 1990). To avoid water, mucins crosslink via disulfide bonds and form the viscoelastic matrix gel along with other biomolecules (Figure 1). To date there are 19 identified human mucin genes, MUC1, 2, 3A, 3B, 4, 5AC, 5B, 6-9, 11-13, and 16-20. However, the only MUC gene products that have been identified as the major gel-forming mucins in respiratory secretions from healthy individuals are MUC5AC and MUC5B.

MUC5AC is expressed in goblet cells, while MUC5B is expressed in mucosal cells of the submucosal gland (Kreda et al. 2012). MUC5AC is produced in limited quantities at baseline levels; however, during cellular inflammation, production is increased (Hauber et al. 2006). MUC5B, on the other hand, is produced constitutively, but, its production rate is altered during inflammation. Henke and colleagues used confocal microscopy data to assess the amount of mucin-like glycoprotein and DNA in CF sputum and found more mucin in bronchitis sputum and a higher amount of DNA in CF sputum. They then analyzed the gel-forming mucins, MUC5AC and MUC5B via western blots to quantify mucin in sputum from CF patients and normal individuals and found that there was a 70% decrease in MUC5B and a 93% decrease in MUC5AC in CF sputum. (Henke et al. 2004).

MUC5B is the most important gel-forming mucin in airway mucus and is required for mucociliary clearance. MUC5B is necessary for airway homeostasis and anti-bacterial defense (Evans et al. 2015). Hypersecretion of MUC5AC and MUC5B have been shown to contribute to pulmonary diseases such as COPD. In general, hypersecretion has been identified as a common denominator in many patients with pulmonary diseases such as CF and COPD (Samet et al. 1994). Interestingly, the

molecular components of mucus vary between the different disease states, which is believed to be due in part to the different expression levels of the two major gel forming mucins MUC5AC and MUC5B present in each disease state. Hypersecretion of mucus can be targeted via therapies that focus on inhibiting mucin synthesis, by reducing the expression of the gel-forming MUC5B gene, and/or inhibition of mucin secretion into the airways (Ha et al. 2016).

Section 1.2 Cystic Fibrosis Mucus

CF is a heavily researched genetic disorder. Symptoms of CF are salty tasting skin, mucus with increased viscosity, lung infections and inflammation, and shortness of breath to name a few. It is an inherited autosomal recessive disease and a case where an individual must inherit two copies (1 copy per parent) of the defective CFTR gene. The individual will only get CF if both parents have a copy of the defective CFTR gene (Zhou et al. 2008). The most common CF mutation is $\Delta F508$, in which three nucleotides that comprise the codon for phenylalanine at position 508 are deleted. Consequently, an individual with this mutation will produce an aberrant CFTR protein that will remain in the endoplasmic reticulum.

CFTR is an ion channel and the most predominant anion conductance in epithelial cells. Patients with CF have deficient Cl^- and HCO_3^- secretion from airway epithelial cells due to the loss of CFTR. Sodium is also hyper-absorbed through ENaC, and this defective anion transport alters osmosis and in result causes mucus dehydration (Gianotti et al. 2015).

CFTR is vital for the production of sweat, mucus and digestive fluids. When CFTR is non-functional, bodily secretions become abnormally thick and acidic (Kreda et al. 2012). The build-up of viscous mucus is believed to be contributed to chronic infection, inflammation, and respiratory disease, which ultimately leads to mortality in patients with CF (Lyczak et al. 2002). A characteristic of CF is accumulated mucus plugs located in the airways, liver, intestines, pancreas, reproductive organs and other mucus-producing exocrine glands (Nicholson et al. 2002). However, the main characteristic of the CF pancreas is a failure to secrete bicarbonate, so pancreatic enzymes stay in the pancreas and digest it, Although CFTR is expressed in multiple organs, pulmonary disease now is the major cause of illness and death in patients with CF (Ermund et al. 2015). In organs affected by CF, surprisingly, no common mucin compositional changes have been identified (Mantle et al. 1990). Currently, the most widely accepted hypothesis in CF is that the lack of chloride secretion and sodium hyperabsorption leads to mucus dehydration. New studies have emerged suggesting that cigarette smoke leads to the inhibition of CFTR, resulting in dehydration of the airway surface liquid.

Despite all of the advancements in the field, there is still no cure for CF. Current therapies have focused on decreasing the symptoms such as inflammation and infection, and on decreasing the viscosity of the mucus layer. Lung infections are treated with antibiotics that can be inhaled, ingested, or injected, such as azithromycin, as well as other therapies such as hypertonic saline and salbutamol (Heijerman et al. 2009). Researchers have also looked at other ion channels on the apical surface of the

airway epithelium for potential therapies for CF, such as the Ca^{2+} activated chloride channel.

Section 1.3 pH Effects on Mucus

Respiratory mucus is found to be a barrier to the penetration of hydrogen ions into the surrounding tissues (Holma et al 1985). Numerous elements within the airway depend on the regulation of pH to carry out their normal functions such as antimicrobial activity, rheological characteristics of gel-forming mucins, mucociliary clearance, and protease activity. For example, the average pH range of eye mucus is 7.5, Nasal mucus 6-8, saliva 6-9, gastrointestinal mucus 1-7 and vaginal mucus 3-9 (Wang et al. 2013).

Defective CFTR leads to infection and inflammation in the ASL due to the excessive amount of pathogens that become trapped. Since the activity of ASL antimicrobials may be additive or synergistic (Singh et al., 2000), changes in pH may disrupt these cooperative interactions. The most common bacteria that infects the ASL and triggers CF morbidity is *Pseudomonas aeruginosa*. It has been well established that when CFTR is absent, airway mucus properties are altered. Loss of CFTR has resulted in alterations in pH, leading to an increase in bacterial colonization, increase in mucus viscosity, and decrease in mucociliary clearance (Berkebile et al. 2014).

HCO_3^- was hypothesized to be critical in the formation of normal mucus by its ability to sequester Ca^{2+} from condensed mucins being discharged from cells. Chen et al. demonstrated that mucin diffusivity increases as a function of HCO_3^- (Chen et al. 2010). Their data proved that HCO_3^- enhances mucin swelling and hydration by reducing Ca^{2+} cross-linking in mucins, thereby decreasing its viscosity and likely

increasing its transportability. In addition, HCO_3^- can function as a Ca^{2+} chelator like EGTA to disperse mucin aggregates. This finding suggests that poor HCO_3^- availability in CF may explain why secreted mucus remains aggregated and more viscous in affected organs. This was the first piece of evidence showing that HCO_3^- modulates the rheological properties of mucins released from living cells (Chen et al. 2010).

Coakley and colleagues proved that deficient CFTR alters bicarbonate secretion which in turn leads to a decrease in ASL pH. This observation was reported in human primary airway epithelial cells (Coakley et al., 2003), cultured sub-mucosal glands (Song et al., 2006), condensate from human patients (Tate et al., 2002), and porcine tracheal ASL (Pezzulo et al., 2012). Studies have also shown that the on-set of ASL pH alterations is age dependent. McShane and co-workers observed no differences in ASL pH between people with CF and non-CF controls aged 3 years or older (McShane et al., 2003).

Antimicrobial peptides and proteins are key components of the innate immune response, that help determine whether exposure to a pathogen leads to a disease state. Their reaction to infection is very quick, within minutes to hours of infection. Genetically modified pigs have become a very popular model system for CF. Studies have shown that immediately following birth, the ASL of non-CF pigs is able to kill bacteria, however the ASL of CF pigs is unable to kill bacteria (Pezzulo et al. 2012). This observation could be due to the change of ASL pH in CF pigs, and the shift in pH is possibly not suitable for antimicrobials; when they altered the pH of CF ASL to see if they could rescue the non-CF phenotype, they observed that increasing the pH restored bacterial killing in CF airways (Pezzulo et al. 2012).

Recently, investigators have begun to look more closely at short palate lung and nasal epithelial clone 1 (SPLUNC1), which is a pH sensitive secreted peptide that regulates ENaC activity. Studies have shown that in an acidic environment, such as the CF ASL, SPLUNC1 becomes deactivated and ENaC is no longer regulated, causing a hyperabsorption of sodium into the ASL.

While secreted and tethered mucins are key factors in airway defense, the abnormally viscous mucus found in the CF airways impairs mucociliary clearance and contributes to airway obstruction and bacterial colonization. Of note, mucins become more viscous at acidic pHs (Bansil et al. 2013), and this may further contribute to the thickened mucus that characterizes CF lung disease. Jayaraman et al., reported that CF submucosal gland secretions are more viscous than non-CF secretions, though they did not detect a difference in pH between CF and non-CF secretions (Jayaraman et al. 2001). Recently, Gustaffson and colleagues reported that the addition of HCO₃⁻ to mucus from the small intestines of CF mice reduced both mucus density and its adherence to intestinal epithelium (Gustafsson et al., 2012). This is consistent with previous data from Chen et. al that demonstrated that HCO₃⁻ reduces aggregation of porcine gastric mucins (Chen et al., 2010).

There is evidence that reductions in pH lead to reduced ciliary beat frequency in human airways (Luk and Dulfano, 1983). While the mechanism behind this is not fully understood, pH has recently been linked to the regulation of ASL volume and with a reduction in pH favoring a reduced ASL volume (Garland et al., 2013). Therefore, in CF, a reduction in ASL volume may impair ciliary beating; the altered viscosity of CF mucus

may further reduce the ability of cilia to clear mucus from the airways (Luk and Dulfano, 1983).

In summary, the airways have an intrinsic host defense system that consist of coughing, the barrier properties of the epithelium, secreted mucus and antimicrobials, mucociliary clearance, and phagocytic cells (Berkebile et al. 2014). While changes in ASL pH is not the only contributor towards increased susceptibility of the CF airways to bacterial infection and colonization, they are a likely contributor to the early host defense defect (Berkebile et al. 2014). Understanding how changes in pH impact mucosal immunity may lead to new therapies that can modify the ASL environment, improve airway defenses, and alter the disease course.

Section 1.4 Ca^{2+} Effects on Mucus

There have been a lot of controversial findings regarding the effects of Ca^{2+} on mucus. One study proposed that Ca^{2+} ions act as cross-linkers between mucin molecules (Raynal et al. 2003), however, Forstner et al., detected no evidence of precipitation, gelation or polymerization of mucin glycoprotein in the presence of Ca^{2+} (Forstner et al. 1976). They used a purified rat intestinal goblet cell mucin as a model mucin to investigate the effects of millimolar additions (1-25 mM) of CaCl_2 on the physical properties of mucin. They found that CaCl_2 (8-15 mM) caused a 15-33% decrease in viscosity and a 20-30% decrease in solubility of the mucin. Moreover, solubility changes were reversed by the addition of 20 mM EDTA. They also demonstrated that Ca^{2+} alters the 3-D structure of mucin by changing its density and excluding water. This data suggested that when Ca^{2+} is present, mucin becomes denser, potentially due to the loss of intramolecular water.

Several studies have led to the suggestion that Ca^{2+} forms complexes with mucus. Deman et al., proposed that Ca^{2+} forms ionic bridges between porcine gastric mucin (PGM) molecules via the carboxylic acid groups of sialic acid moieties (Deman et al. 1973). However, other investigations on tracheal and cervical mucus glycoprotein indicate that these mucins are not dependent upon sialic acid for their rheological activity. The existence of some sort of calcium-mucin complex has been proposed. It has been noted further that Ca^{2+} causes aggregation of a variety of glycoproteins, resulting in solutions of high turbidity, and produces abnormal patterns in electrophoresis (Deman et al. 1973).

Gibson et al., established that Ca^{2+} is abundant in secretory granules that secrete mucins and Ca^{2+} levels are increased in CF patients. Failure to remove Ca^{2+} ions from the mucosal surface may lead to an abnormally thick, dehydrated mucus. It is hypothesized that elevated concentrations of Ca^{2+} within glycoprotein secretions of patients with CF may substantially increase the density and insolubility of mucins, promoting the formation of muous “plugs”. Experimental results indicate that an increase in extracellular Ca^{2+} concentration can drastically decrease the diffusivity of the newly released mucin, resulting in extremely slow rates of swelling and a mucus gel that remains thick for long periods of time (Verdugo et al., 1987). These findings helped spark the idea that Ca^{2+} plays a role in modifying conformational changes of mucin glycoproteins.

A study by Forsrner et al., suggested that Ca^{2+} is responsible for aggregation of small salivary proteins, but no studies have identified whether Ca^{2+} changes the physical properties of purified mucin macromolecules (Forstner et al. 1976). A cause

and effect relationship between the control of the ionic environment on the mucosal surface and the viscoelastic properties of mucus has been suggested. Results have indicated that mucus hydration is governed by a Donnan equilibrium process; which is explained as the behavior of charged particles near a semi-permeable membrane that sometimes fail to distribute evenly across the two sides of the membrane. It has been shown that HCO_3^- chelates mucus-bound Ca^{2+} . The effects of Ca^{2+} on mucin has been heavily studied using dynamic light scattering and fluorescence recovery after photobleaching (FRAP). Varma and colleagues used dynamic light scattering to show that Ca^{2+} induces a contraction or folding of the mucin chains (Varma et al. 1990). This effect could be physiologically important because the presence of high levels of extracellular Ca^{2+} in CF patients, may lead to the formation of a contracted and hence more densely entangled mucin gel, with a high elastic modulus.

Chapter 2 THE EFFECTS OF CALCIUM ON MUCUS COMPOSITION AND RHEOLOGY

Introduction

Raynal et al. used fluorescence recovery after photobleaching (FRAP), a method used to measure diffusion through a tissue/cell, to demonstrate that the addition of CaCl_2 into saliva (with high levels of MUC5B) alter the viscosity of saliva and increased its molecular weight (Raynal et al. 2002). However, when a Ca^{2+} chelator, EGTA was introduced to the saliva, the viscosity as well as the molecular weight decreased (Raynal et al. 2002). These findings led us to hypothesize that Ca^{2+} may play a role in the development of viscoelastic macromolecular properties in mucus.

Taking into consideration previous studies, we utilized purified MUC5B, bovine submaxillary mucin (BSM), and PGM as our mucin model systems. BSM originates from mucus secretions from salivary glands located under the floor of the mouth and PGM originates from the pig gastrointestinal tract. They are similar in structure to human mucins and conserve most of the same proteins, including mucin gene products MUC5AC in PGM, and MUC5B and MUC5AC in BSM, as well as glycoproteins. We utilized the techniques of confocal imaging, macrorheology, and fluorescent single particle tracking, to better understand the effects of Ca^{2+} on mucin rheology. Utilizing these techniques made our findings significant because it will allow for a deeper understanding of mucus rheology, thus leading to the potential development of

chelators as therapeutics to decrease viscoelastic properties in individuals with pulmonary diseases. We set out to investigate how extracellular ions such as Ca^{2+} influence mucin rheology and mucociliary transport.

Section 2.1 Methods

Reagents: 1M CaCl_2 solution was obtained from Sigma-Aldrich catalog #21115, and diluted to 1, 2, 4, 10 and 100 mM when added to mucin models. H300 and MUC5AC antibodies were purchased from Santa Cruz. BAPTA, AM, cell permeant chelator was obtained from ThermoFisher Scientific. CF633 wheat germ agglutinin was purchased from Botium catalog #29022-1 and was diluted to 1-2 mg/ml before use. Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Confocal microscopy: Fluorescent probes were added to the mucin samples and were incubated overnight at 4°C in the dark. Following incubation mucin samples were added to dialysis tubing and buffered against 1X PBS overnight at 4°C. The next day samples were incubated with appropriate ion/molecules or Ca^{2+} chelating reagents (Sigma) overnight at 4°C. The sample was then deposited on a glass slide for confocal imaging (Leica SP5; 40X oil immersion lens).

Fluorescent particle tracking: 1 μm fluorescent tracer particles were diluted 1:1000 from their stock solution (2 wt. %) into mucin samples. Following mucin treatment with ions and chelators, 5 μl of the mucin sample was sealed between a glass microscope slide and coverslip with two sheets of parafilm acting as a spacer. The sample holders were sealed on parafilm to minimize sample evaporation. Tracer particle

movement was recorded at 60 frames s⁻¹ for 30 s with a Flea3 grey scale camera (Point Grey, Richmond, Canada) mounted on a Nikon Eclipse TE2000-E inverted microscope at a magnification of 40x. The tracer particle displacement as a function of time was quantified using Video Spot Tracker software (Center for Computer Integrated Systems for Microscopy and Manipulation, University of North Carolina at Chapel Hill). For each condition tested, tracer particle displacement was measured in 15 different viewing areas of three separate mucin samples, resulting in an ensemble averaging of at least 200 particles per treatment.

Cytation 5 imaging reader: 20 µl of sample was added per well in duplicate in a 384 well plate. Alexa fluor conjugate of WGA 594 was read (excitation, 590 nm; emission, 618 nm). Images were taken with 4x lens in each well. Exposure was set to 10 LED, 8 gain and autofocus.

Statistical analyses: All data were checked for homogeneity of variance and analyzed using ANOVA parametric. All values are expressed as means standard error, and was set to 0.05. n refers to the sample size. All analyses were conducted using Prism 5 (GraphPad, La Jolla, CA) software.

Section 2.2 Results and Discussion

Gastric mucin is very similar to other mucins. They are very high molecular weight glycoproteins that are composed of approximately 80% polysaccharides. Unlike airway mucus that is composed of the gel-forming mucins MUC5AC and MUC5B, gastric mucus is composed of MUC5AC, MUC1 and MUC6. Of the 19 identified mucin genes the sequences of homology have been fairly similar. They all contain non-

glycosylated and glycosylated domains. The mucins have tandem repeating sequences of serine, threonine, and proline that are linked to the O-glycosylated portion of the molecule (Figure 1A,B). The length and number of serine, threonine, and proline repeats vary for the different mucin genes and species. For instance, MUC5AC contains 66–124 repeats of 8 amino acids (Bansil et al. 2013). In all secretory mucins, the glycosylated domain is located in the center of the proteins and is flanked by cysteine rich domains and a cysteine knot domain, with non-repeating sequences at the C-terminal and by domains similar to the von Willebrand Factor (vWF) C, D domains, at the N-terminus (Figure 1C,D). Mucins are heavily cross-linked to form a mucin gel mesh network that is used to trap inhaled pathogens for mucociliary clearance (Figure 1E).

We first wanted to determine the organizational pattern of the polysaccharides within the bovine submaxillary mucin model system. We hypothesized that the addition of Ca^{2+} would alter mucins organizational properties of mucins due to calcium's ability to increase crosslink mucins. We used wheat germ agglutinin conjugated to Alexa633 (WGA 633). WGA is a lectin protein that binds to N-acetyl-D-glucosamine, a derivative of glucose, and also binds to sialic acid. We treated 2.5% BSM with 2mg of WGA 633 overnight in the presence or absence of 2 mM CaCl_2 (Figure 2). We observed increased mucin clusters at a ratio of 2 to 1 when CaCl_2 was present in the mucin (Figure 2B). Although we saw changes in cluster number at higher magnification, we observed no difference in gross mucin structure in the presence or absence of CaCl_2 . These data suggested that CaCl_2 may affect mucin structure.

We next wanted to investigate whether or not MUC5B was affected by the addition of CaCl_2 (Figure 3). We treated 2.5% BSM with WGA 633 overnight and then

with mouse H300 antibody, which binds to MUC5B. In the control experiment, there was little aggregation of MUC5B. However, when 4 mM CaCl_2 was present, we observed co-localization between WGA and MUC5B. We wanted to see whether or not we could reverse this effect if we added the Ca^{2+} chelator EGTA. With the addition of 30 mM EGTA, the co-localization phenomena was reverted back to that of the control (Figure 3A). We quantified the co-localization between WGA and H300 fluorescence using Pearson's r correlation test, which measures the linear correlation between two variables, giving a final value between +1 and -1, where 1 is total positive correlation, 0 is no correlation, and -1 is a negative correlation. There was a significant difference in correlation between WGA and CaCl_2 , with the average r being 0.8. When EGTA was added, co-localization between WGA and MUC5B remained between some molecules, but most had reverted back to normal (Figure 3B).

In another experiment, we used a Cytation 5 imaging plate reader to measure the fluorescence intensity of WGA-633. We used this device to test whether or not fluorescence intensity was perturbed in the presence of different concentrations of CaCl_2 ranging from 100 mM CaCl_2 and 30 mM EGTA (Figure 4). The WGA fluorescence intensity was independent of the CaCl_2 concentrations (Figure 4A, B). Additionally, we did notice there was a change in WGA fluorescence intensity increase with the addition of EGTA but it was not significant (Figure 4C, D).

With data suggesting that Ca^{2+} may alter the cross-linking of mucins, we next wanted to look at the rheological effects of Ca^{2+} on mucins. We began our experiments by utilizing the macrorheology of PGM in the presence of Ca^{2+} . The rheological properties of a material describes how the material responds to an applied stress. At the

chemical level, mucus is an integrated structure of biopolymers. Its physical behavior is complex (non-Newtonian), highly variable and exhibits both viscous and elastic properties. Rheological measurements, including viscosity (resistance to flow) and elasticity (stiffness), are often used together to describe the consistency of mucus (Lai et al., 2009). The rheological properties of mucus varies as a function of shear stress, time scale (rate) of shearing, and length scale. Changes in the rheological properties of mucus may greatly affect its ability to function as a lubricant, selective barrier and mucociliary clearance (Lai et al., 2009).

We began the macrorheology experiments by observing the effects of different concentrations of CaCl_2 on PGM complex viscosity properties. We performed a frequency sweep test, in which a frequency is varied while the amplitude of the shear stress is kept constant. When PGM was treated with CaCl_2 overnight and then applied to a frequency sweep, we did not notice a change in viscosity at 1 mM or 100 mM CaCl_2 . However, we did see a significant increase in the complex viscosity of the mucin in the presence of 10 mM CaCl_2 (Figure 5A). We repeated the same experiment but with the addition of 30 mM EGTA or 50 mM BAPTA, Ca^{2+} . However, we did not see a change in viscosity over any CaCl_2 concentration (Figure 5B). However, when we compared 1mM CaCl_2 to 10 mM CaCl_2 + 30 mM EGTA and 10 mM CaCl_2 + 50 mM BAPTA, we saw a significant decrease in complex viscosity when Ca^{2+} was chelated (Figure 5C). We repeated this experiment using MgCl_2 to analyze whether or not this phenomenon was limited to CaCl_2 or was universal. We did not observe any significant increase in complex viscosity in the presence of MgCl_2 (Figure 6).

Microrheology is a measurement of viscoelasticity at the nanoscale level. In contrast to macrorheology, which provides averaged measurements of physical properties, microrheology can measure heterogeneity within a samples with high spatial resolution (Lai et al., 2008). Particle tracking, is firmly established as a technique for characterization of both diffusive transport properties and linear viscoelasticity (Mellnik et al. 2014). In essence, microrheology allows for characterization of both the viscosity and elasticity of biological fluids, and can take into account both contributions from the fluid within the biopolymer network as well as the network mesh itself. Thus, microrheological studies are important for characterizing the local mechanical properties of biological fluids that are often not detected on the macro scale (Lai et al., 2008).

We used 2.75% purified MUC5B and the fluorescent single particle tracking technique to study the effects of CaCl_2 on mucin rheology. Mucin was treated with 4 mM CaCl_2 and varying concentrations of EGTA overnight. When compared to the 4 mM CaCl_2 only, the mean squared displacement (MSD) decreased in a dose dependent manner in the presence of higher concentrations of EGTA (Figure 7). We separated the data of interest into another graph (Figure 7B) and observed that when 4 mM CaCl_2 was added to the mucin sample, there was a slight increase but not significant in viscosity properties in comparison to the bead only control. When EGTA was introduced, we saw a decrease in the MSD in a dose dependent manner. We quantified the slope of the first and last points, (Figure 3A) and saw that there was a large change between 4 mM CaCl_2 only, compared to CaCl_2 in the presence of EGTA. This data, along with the macrorheology data, suggests that CaCl_2 alters the viscosity of mucins.

CHAPTER 3: THE EFFECTS OF MUCUS pH ON RHEOLOGY

Introduction

Previous studies suggested that pH could affect the physical properties of mucins, mucus, and human sputum (Ehre 2014). To compliment this data, we decided to perform a pH titration curve on PGM. Garland et al. demonstrated that the loss of CFTR markedly reduces airway epithelial HCO_3^- secretion and ASL pH in CF patients (Garland et al. 2013). This observation led us to test whether or not the increased viscosity is due to pH or HCO_3^- . It is well known that CF is a disease of disrupted epithelial electrolyte transport (Quinton 1990). The two key substances that are disrupted in CF are mucus and HCO_3^- . HCO_3^- is a salt, a base, and a chelator and is believed to be required for gel-forming mucins to form normal mucus. It has been hypothesized that normal mucin formation and release requires CFTR dependent HCO_3^- secretion, to potentially sequester Ca^{2+} from condensed mucins as they expand from their secretory vesicles (Yang 2013). As described by the Henderson-Hasselbalch equation, pH, HCO_3^- , and CO_2 are all related. Thus, because HCO_3^- is the major buffer, its secretions are very important and tightly regulated.

Section 3.1 Methods

Mucin Sample preparation: Porcine Gastric Mucin Type III, sialic acid 0.5-1.5%, partially purified powder (PGM: MUC5AC); (Sigma-Aldrich) were dissolved in 1X PBS at concentrations that mimic those seen in human respiratory mucus.

pH readings: pH was recorded using the ORION II Medical Measurement System and software. The catheter was calibrated in pH 4.0 and 7.0. After calibration, pH of the PGM was checked and recorded and was raised to pH 8.0 with 1 M HCl. pH was then determined utilizing titration curves and adjusting pH accordingly using 100 mM NaOH. 1 M NaHCO₃ was diluted accordingly in PGM to achieve 5 mM and 20 mM.

Macrorheology: We used a 40 mm 1.012o cone plate, and a Peltier plate steel on a Trios rheometer for all macrorheology data. Frequency sweeps were performed in triplicates at 23oC, at 1% strain and an angular frequency of 0.05 to 20 rad/s. Amplitude sweeps were performed in triplicates at 23oC, at 0.01 to 10% strain, at an angular frequency of 1rad/sec and 10rad/sec.

Section 3.2 Results and Discussion

We first determined the amount of protons required to 50mg/ml and 100mg/ml PGM to raise the pH the 100mg/ml PGM to pH 4-8. The average pH of PGM is pH 6.5. We added 1 M HCl and 100 mM NaOH to conduct a titration curve from pH 8 to pH 4. We then added 0 HCO₃⁻, 5 mM HCO₃⁻, and 20 mM HCO₃⁻ and conducted another titration curve from pH 8 to pH 4.

After we determined how much 100 mM NaOH was needed to achieve a certain pH in the presence and absence of HCO₃⁻, we then made an aliquot of each sample at

a given condition at the respective pH levels over the range of 4-8 to analyze the changes in viscosity due to, pH changes in HCO_3^- . Samples were made and titrated at room temperature. Therefore, CO_2 was not taken into consideration for this set of samples. In the 0 mM and 5 mM HCO_3^- 50 mg/ml (5%) PGM samples, we saw no significant correlation between pH and complex viscosity (Figure 8A-B). However, in the 20 mM HCO_3^- PGM sample, we saw that as pH increased, the complex viscosity decreased and vice versa (Figure 8C).

We next tested whether or not a higher concentration of PGM (100 mg/ml; 10%) in the presence of HCO_3^- would induce a stronger correlation between pH and complex viscosity. 10% PGM did not show a trend between pH or complex viscosity, under HCO_3^- free conditions (Figure 8D). However, 10% PGM with either 5 mM HCO_3^- and 20mM HCO_3^- showed a slight but not significant trend between pH and complex viscosity (Figure 8E-F).

Discussion

In healthy individuals, mucus production, secretion, and clearance play an important role in innate defense of the airways. However, in patients that have CF pulmonary disease, where the CFTR gene, is mutated leading to defective CFTR protein causes a disruption in airway ion transport, leading to reduced ASL volume and decreased pH. This in turn causes an overly abundant and viscous mucus, resulting in decreased mucociliary clearance, increased bacterial infections and lung damage (Figure 9). In instances where the mucus attaches to the epithelium, increased ciliary beat frequency may not be enough to clear pathogens from the airways and external therapeutics are required.

Previous work by Ambort and colleagues, suggested that in the intestines purified MUC2 N-terminus formed aggregates at high Ca^{2+} and low pH, aggregates which could be dissolved by Ca^{2+} chelation and raised pH (Ambort et al. 2012). We wanted to determine whether or not airway mucins displayed a similar cluster effect under the influence of Ca^{2+} . Based on our results we observed that in the presence of Ca^{2+} , mucins formed clusters at a ratio of 2 to 1 in comparison to mucins without CaCl_2 . However, when we tested to see whether or not CaCl_2 disrupted the binding ability of mucins to molecules such as WGA 633, we did not observe a change in fluorescence intensity in the presence or absence of CaCl_2 and EGTA. Physiologically, HCO_3^- would be the natural chelator of extracellular Ca^{2+} . This data suggest that Ca^{2+} plays a role in the crosslinking of mucins, by changing the organizational pattern and increasing mucin aggregation, but without altering the binding affinity of mucins to other molecules.

It has been hypothesized that elevated Ca^{2+} within glycoprotein secretions of patients with CF may increase the density of mucins promoting the formation of mucus plugs. Utilizing macrorheology and fluorescence single particle tracking, we observed in the presence of 10 mM CaCl_2 the complex viscosity of both PGM and purified MUC5B were increased significantly. This effect, however, was reversed with the addition of 30 mM EGTA and 50 mM BAPTA. Our data compliments a study conducted by Raynal, who utilized FRAP to observe the effects of CaCl_2 on the complex viscosity of saliva. They saw that the addition of 10 mM CaCl_2 , the viscoelastic properties of saliva increased significantly, in addition to an increase in molecular weight. When EGTA was added to PGM, the complex viscosity was decreased (Raynal et al. 2003). Our work was complimented by several other studies that showed mucins become smaller and denser when CaCl_2 is present (Forstner et al. 1976) and increased Ca^{2+} ion concentrations increased ASL viscosity (Tang et al. 2016).

Aside from extracellular Ca^{2+} , recent studies have suggested that HCO_3^- and pH contribute to increased mucus viscoelastic properties. Our data also support recent studies that have shown that at low pHs, the complex viscosity of PGM increases (Bhaskar et al. 1991). Here, we show via macrorheology that the complex viscosity of PGM increases in a pH dependent manner. That is, as pH increases, the complex viscosity decreases. We also demonstrated that at higher concentrations of HCO_3^- , PGM induce a more linear pH response. Our data proves that higher concentrations of HCO_3^- and lower pH concentrations influence mucin complex viscosity. Continuous development of model systems and techniques to study human mucus, along with more studies targeted towards understanding the underlying binding kinetics of Ca^{2+} to

mucins and its rheological role, as well as the relationship between pH, HCO_3^- and mucus and how it effects mucus flow, will help shed light on future therapeutics for not only CF but numerous pulmonary diseases. Our data suggest that therapeutics found on chelating Ca^{2+} in mucus may help thin the mucus. Furthermore, altered mucus rheology may be a useful biomarker of exposure that can inform us as to both disease progression and the impact of tobacco products on the airways. Therapeutics that will focus on thinning the mucus layer for sufficient and efficient mucociliary clearance.

Figure 1

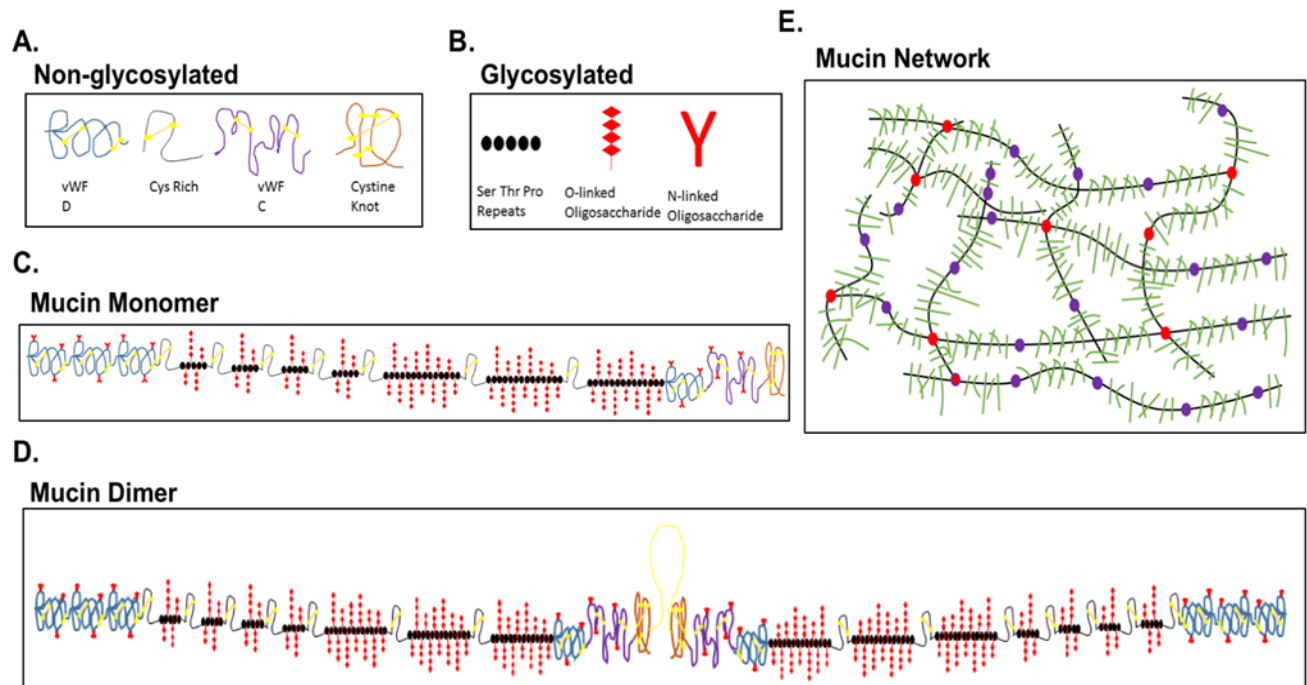


Figure 1. Illustration of mucin domains and gel-forming networks. **A.** Representation of the non-glycosylated domains of mucins as labeled. **B.** Representation of the glycosylated domains of mucins as labeled. **C.** Representation of a mucin monomer. Non-glycosylated and glycosylated domains tether together, with Von Willebrand factor (vWF) domains at the N-terminus and cysteine rich domains at the C-terminus, interconnected with sugars and serine, threonine and proline repeats. **D.** A dimer formed by two monomers linked with a disulfide (S–S) bond connected at the C-terminal a cysteine knot domain. **E.** A representation of a mucin gel network demonstrating crosslinking interactions by color shifting of non-glycosylated domains from purple to red.

Figure 2

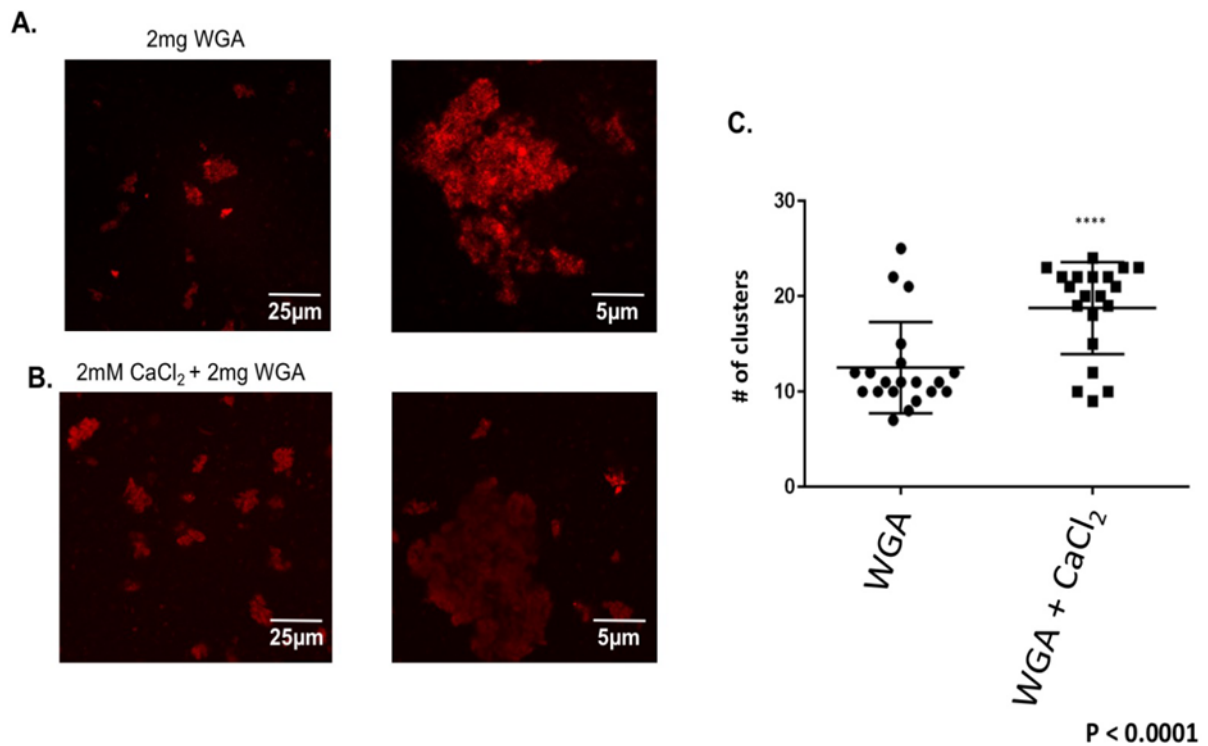


Figure 2. CaCl_2 causes mucin clustering in bovine submaxillary mucus (BSM). **A.** 2.5% BSM was stained with 2 mg/ml WGA 633 and examined by confocal microscopy. WGA labeled all glycoproteins within the mucin (Magnified upper right panel). **B.** 2.5% BSM was treated with 2 mM CaCl_2 and then stained with 2 mg WGA conjugated to Alexa633, dialyzed and examined by confocal microscopy. With the addition of CaCl_2 , we observed larger aggregates of mucin clusters per image field. **C.** An unpaired Student's t-test was used to quantify and compare the number of clusters. $P < 0.0001$. $n=15$.

Figure 3

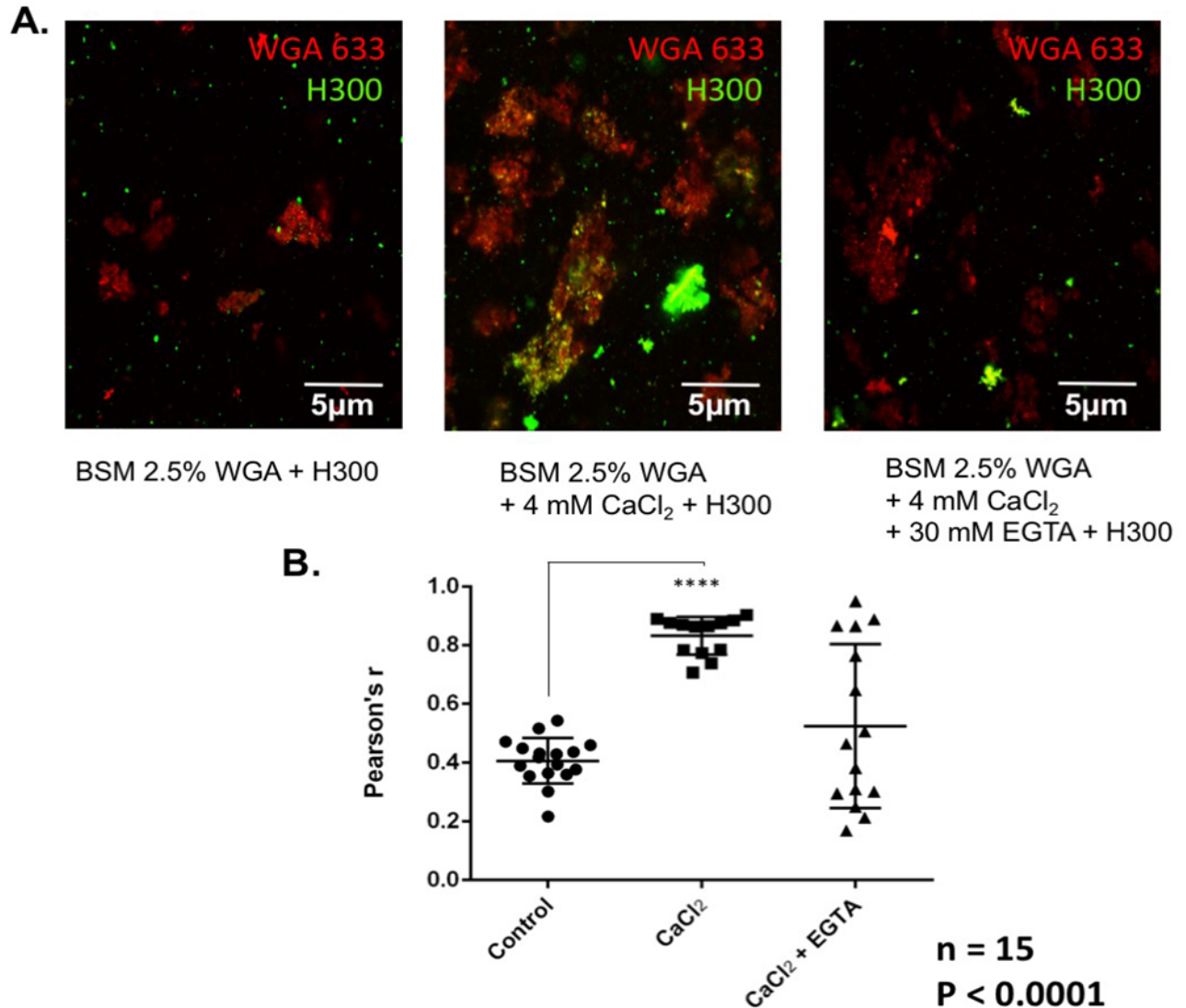


Figure 3. 4 mM CaCl_2 results in the co-localization of MUC5B with WGA in 2.5% BSM and the phenomenon is reversed in the presence of EGTA. A. 2.5% BSM was treated with H300 (MUC5B antibody) and 2 mg WGA. H300 was added to BSM with an Alexa 488 secondary antibody. MUC5B and WGA were present in all conditions. However, in the presence of 4 mM CaCl_2 H300 and WGA co-localized (middle panel). That effect was reversed with the addition 30 mM of Ca^{2+} chelator EGTA (right panel). **B.** Fluorescence intensity was quantified using the Pearson r correlation test. Values near 1 equal strong co-localization whereas 0 represents no co-localization, P < 0.0001. n=10.

Figure 4

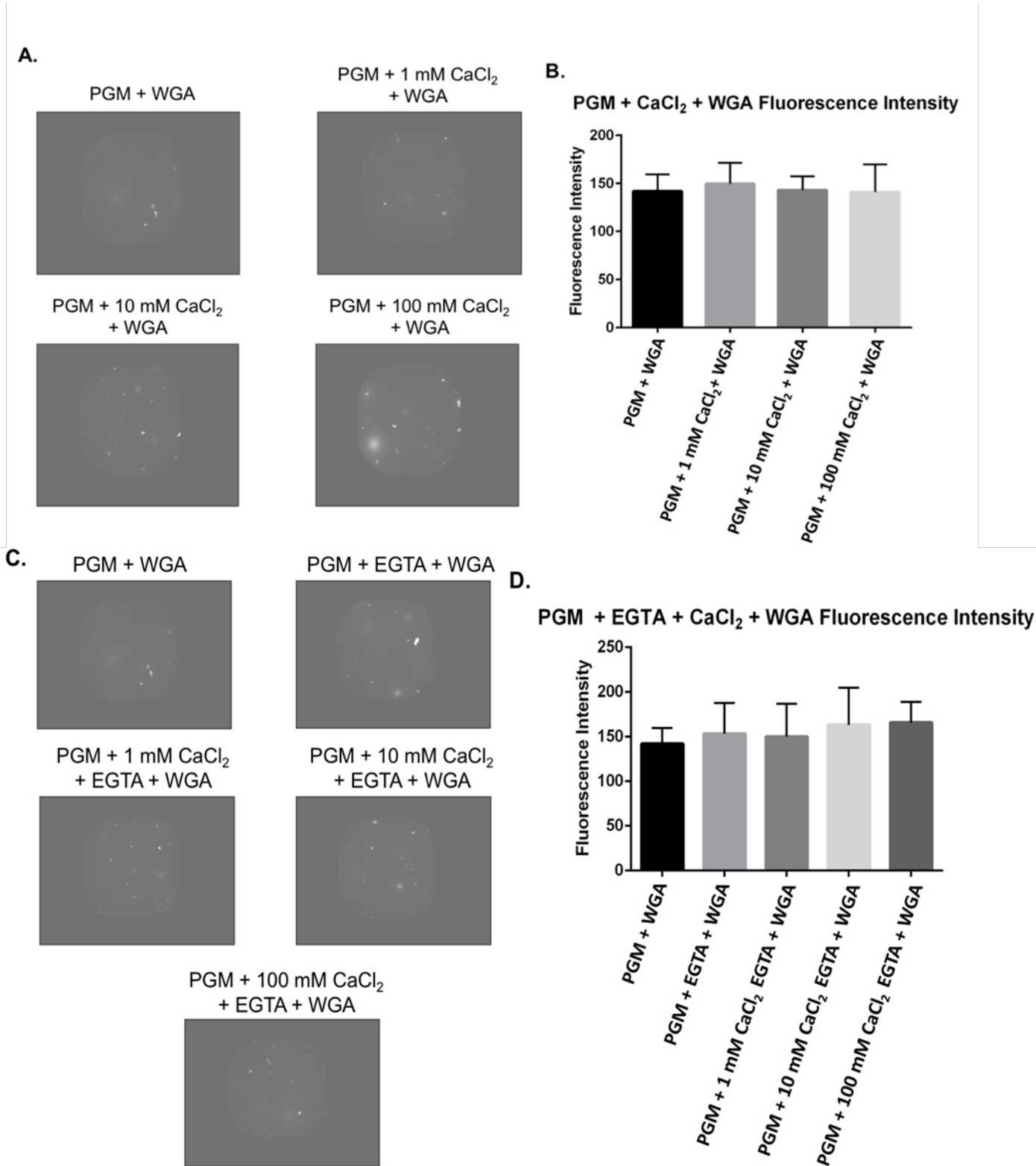


Figure 4. WGA fluorescence intensity is not altered in the presence of CaCl₂ or EGTA. **A.** 5% PGM was incubated with 0.5 mg 594 WGA overnight at 4°C. PGM was then treated with various concentrations of CaCl₂. A Cytation 5 imaging plate reader was used to measure quantitative fluorescence **B.** Quantitative fluorescence was also measured with the Cytation 5 plate reader. **C.** 5% PGM was incubated with 2 mg 594 WGA overnight at 4°C. PGM was then treated with various concentrations of CaCl₂ in addition to 30 mM EGTA. Cytation 5 imaging was used to measure qualitative fluorescence **D.** Quantitative fluorescence was also measured with the Cytation 5 plate reader. All conditions produced a strong fluorescent signal. n=3.

Figure 5

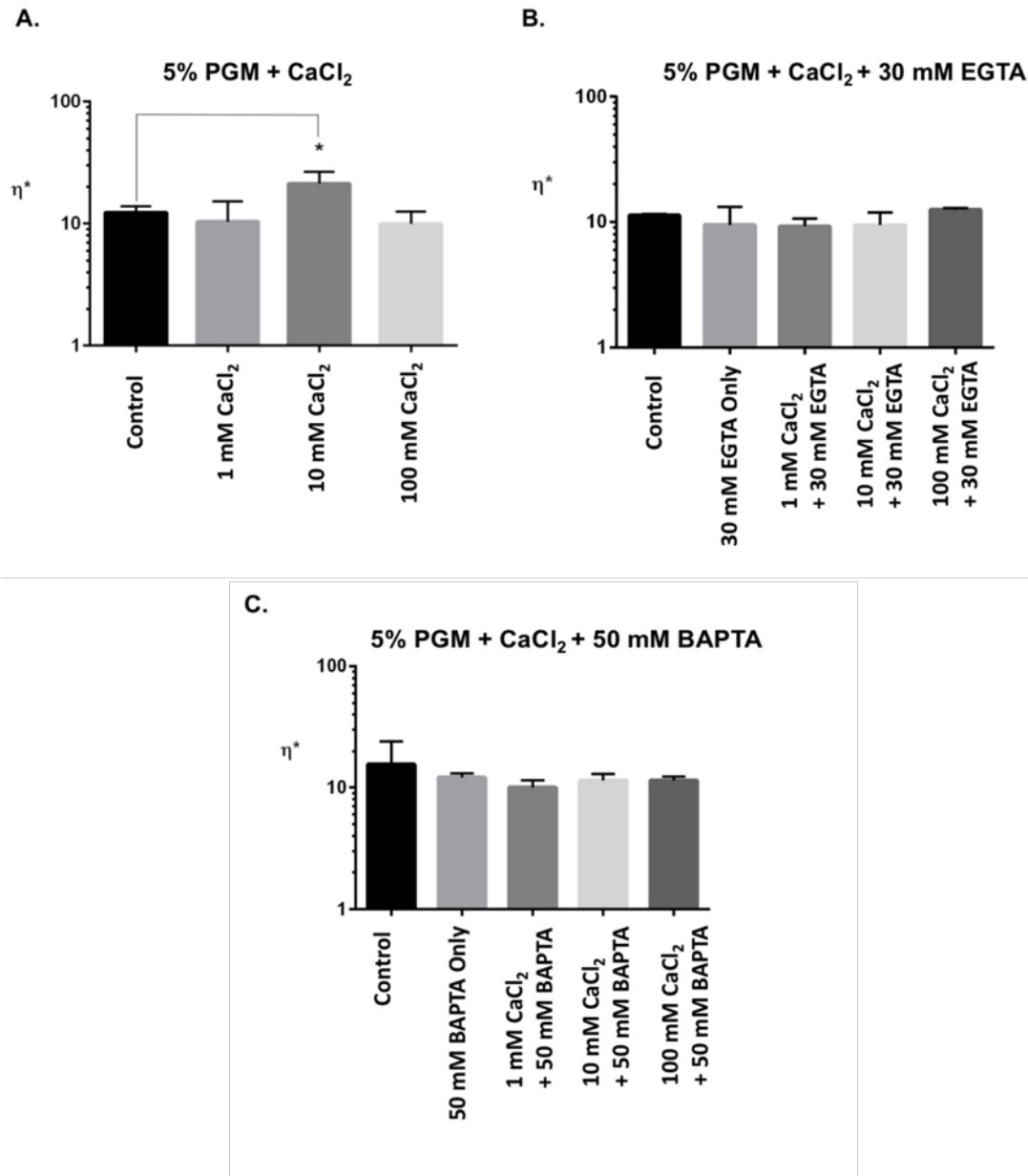


Figure 5. 10 mM CaCl₂ increases 5% PGM complex viscosity and this effect was decreased in the presence of EGTA. A. 5% PGM was treated with 1 mM, 10 mM 100 mM CaCl₂ overnight at 4°C. 300 µl of mucin sample was then loaded onto the TRIOS rheometer and a frequency sweep was performed. 1 mM and 10 mM did not alter mucin viscosity in comparison to the control. However, 10 mM CaCl₂ significantly increased mucin complex viscosity. **B,C.** Ca²⁺ chelators, EGTA and BAPTA were introduced to the PGM and frequency sweeps were performed. The chelators did not decrease complex viscosity in comparison to the control. n=3

Figure 6

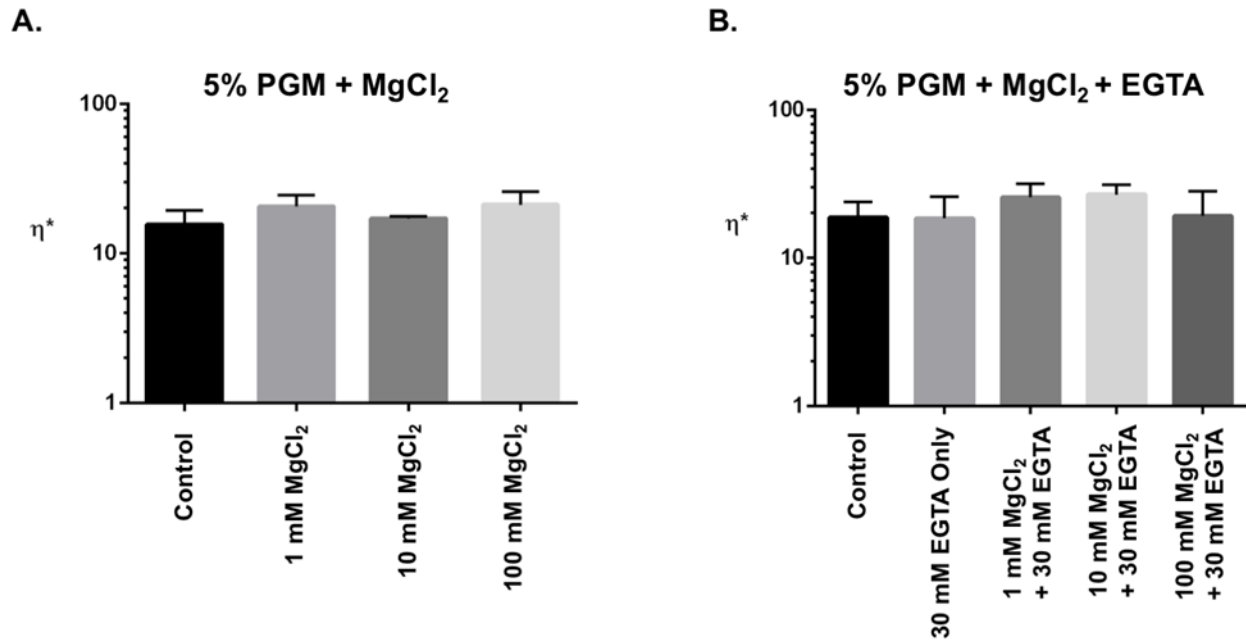


Figure 6. MgCl₂ does not alter the complex viscosity of 5% PGM. **A.** 5% PGM was treated with 1 mM, 10 mM 100 mM MgCl₂ overnight at 40C. 300 μ l of mucin sample was then loaded onto the TRIOS rheometer and a frequency sweep was performed. No concentrations of MgCl₂ increased complex viscosity significantly. **B.** EGTA not only has a binding affinity for Ca²⁺ but magnesium as well. When EGTA was added to PGM and frequency sweeps were performed, EGTA did not alter complex viscosity in comparison to the control. **C.** When comparing all 10 mM MgCl₂ conditions, no significant changes were observed. n=13.

Figure 7

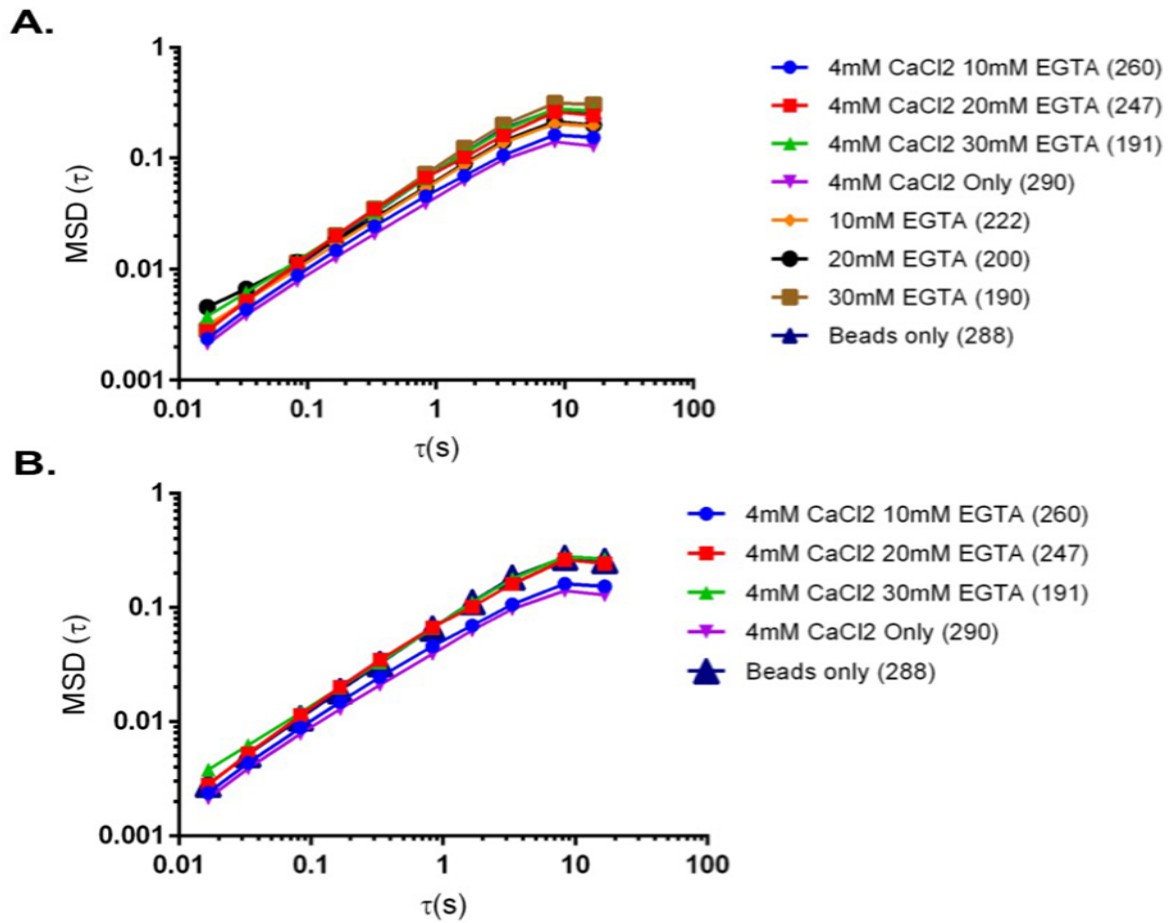
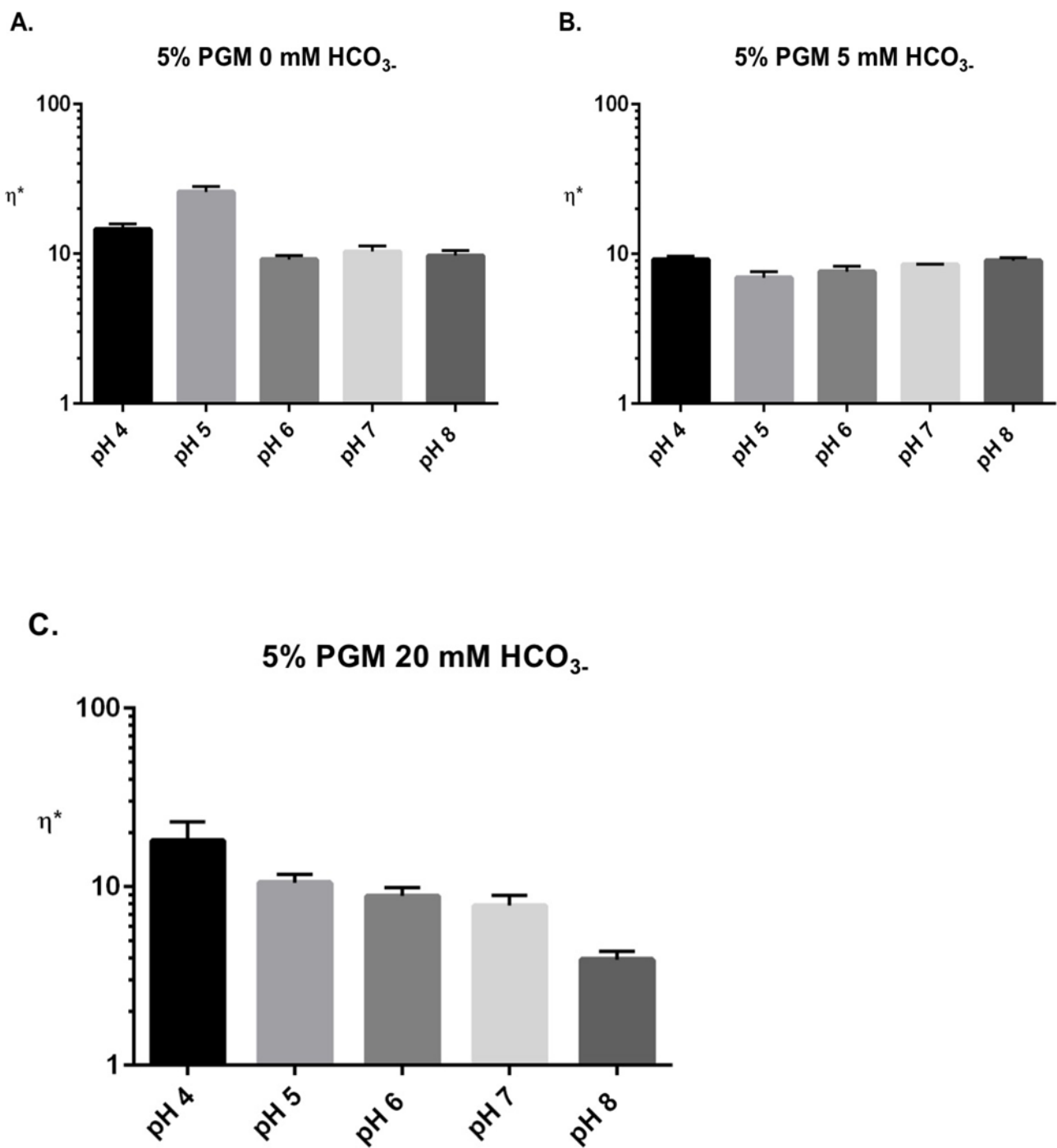


Figure 7. EGTA alters the viscoelastic properties of purified MUC5B in the presence of CaCl₂. **A.** 2.75% purified MUC5B was treated overnight with 4 mM CaCl₂ and varying concentrations of EGTA with or without CaCl₂ and fluorescent single particle tracking technique was applied. The mean squared displacement (MSD) decreased in a dose dependent manner in the presence of EGTA in addition to CaCl₂. **B.** Data of interest from Figure 7A is separated into a simpler graph. When 4mM CaCl₂ is added to mucin there is a slight increase in viscosity properties in comparison to the bead only control. n=3.

Figure 8



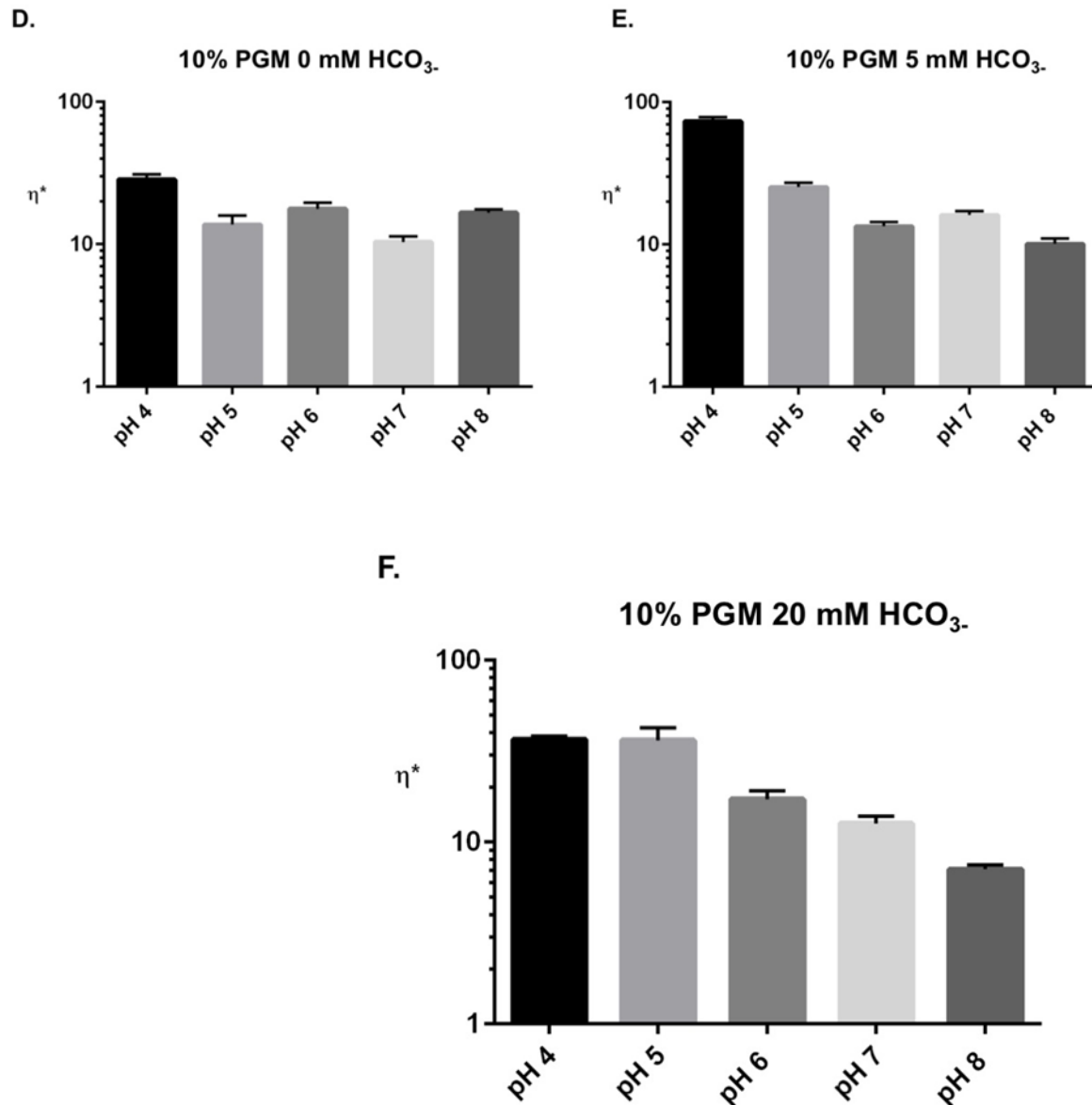


Figure 8. Higher concentrations of HCO_3^- and lower pH increase mucin viscosity.

A. PGM aliquots of each sample at a given condition at the respective pHs 4-8 were made. All samples were adjusted accordingly with 100 mM NaOH over the range of 4-8. Samples were made at room temperature. In the 0 mM and 5 mM HCO_3^- 50 mg/ml (5%) PGM samples we saw no significant correlation between pH and complex viscosity **B.** No change in complex viscosity. **C.** 20 mM HCO_3^- PGM increased complex viscosity in a pH dependent manner. **D-F** We repeated A-C, but with a thicker concentration of PGM 100mg/ml (10%). 10% PGM with 0mM HCO_3^- did not show a trend between pH and complex viscosity. However, 10% PGM with 5mM HCO_3^- and 20mM HCO_3^- altered complex viscosity in a pH dependent trend. *** = $p < 0.001$. $n=3$.

Figure 9

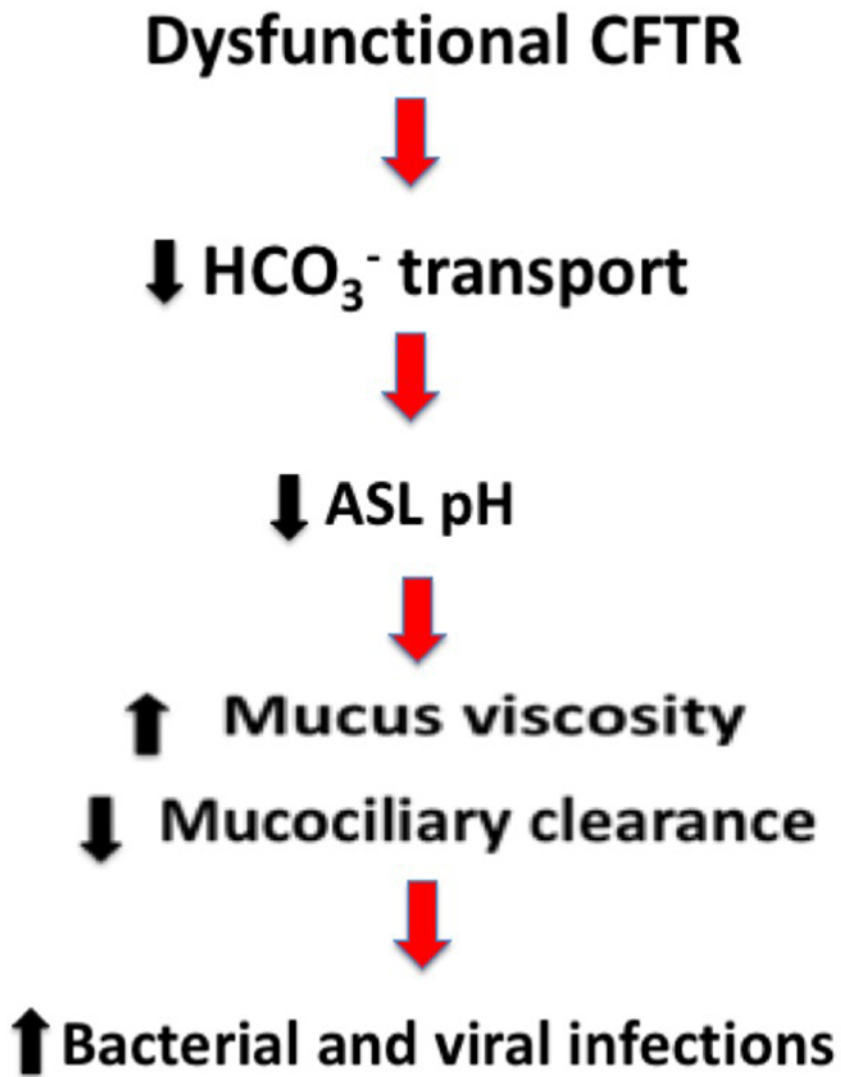


Figure 9. Working model. Defective CFTR due to CF or cigarette smoke exposure, leads to a decrease in bicarbonate secretion, causing a decrease in mucociliary clearance and increased mucus viscosity which is the underlying cause of bacterial and viral infections in pulmonary diseased patients and smokers.

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